Immunity, Vol. 15, 521–531, October, 2001, Copyright ©2001 by Cell Press

Pre-B Cell Receptor Signaling Mediates Selective Response to IL-7 at the Pro-B to Pre-B Cell Transition via an ERK/MAP Kinase-Dependent Pathway

Heather E. Fleming[1] and Christopher J. Paige
Ontario Cancer Institute
Princess Margaret Hospital
University Health Network
Departments of Immunology and Medical Biophysics
University of Toronto
Ontario, Canada

Summary

B lymphocyte development is regulated at multiple checkpoints, mediated by signals originating both inside and outside the cell. Two signaling pathways known to be essential in this process are interleukin-7 (IL-7) and the pre-B cell receptor (pBCR). We have shown previously that these signaling pathways intersect functionally. Specifically, response to low concentrations of IL-7 requires pBCR expression. In this report, we identify the ERK/MAP kinase pathway as a key regulatory component of this response. We propose a molecular mechanism for the selective expansion of pBCR$^+$ precursors and for the culling of inappropriately rearranged pro-B cells.

Introduction

Generation of the B lymphocyte lineage involves the development of early noncommitted hematopoeitic stem cells to mature, Ig-producing B cells. This pathway requires multiple selection checkpoints to ensure appropriate progression through the developmental stages. Among the most important of these selection events are the production of the B cell antigen receptor (BCR) and its precursor, the pre-B cell receptor (pBCR) (Meffre et al., 2000). Surface expression of the pBCR complex requires productive rearrangement of the immunoglobulin heavy chain (HC) and its subsequent pairing with the surrogate light chain (SLC: λ5, VpreB) and the signaling chains Igα and Igβ (Tsubata and Reth, 1990; Venkitaraman et al., 1991). In the absence of a signal derived from an intact pBCR, the cell fails to progress and undergoes apoptotic elimination (Kurosaki, 2000; Osmond et al., 1994). The mechanism responsible for this critical selection step has not yet been defined, despite its essential role in the generation of the B cell lineage. Other pathways have been shown to participate in checkpoints during B cell development. Interleukin-7 (IL-7), a 25 kDa cytokine produced by stromal cells, was identified as a proliferation factor for the B cell lineage (Namen et al., 1988) and has since been shown to promote survival as well. Disruption of the IL-7 signaling pathway prevents the appearance of the pBCR-expressing population in the bone marrow (Hofmeister et al., 1999). However, expression of the *bcl-2* gene in IL-7-deficient cells fails to restore B cell development (Maraskovsky et al., 1998), consistent with other evidence that IL-7 effects differentiation (Corcoran et al., 1996; Cumano et al., 1990; Wei et al., 2000), in addition to its survival and proliferative roles. The most well-characterized signaling consequences of IL-7R dimerization are the activation of PI3K and JAK/STAT proteins (Hofmeister et al., 1999). Additional proteins are beginning to emerge as important members of the IL-7R signaling cascade, including the kinase Pyk2 (Benbernou et al., 2000), which may link IL-7 binding to the Ras pathway (Della Rocca et al., 1997). The Ras/MEK/ERK pathway is believed to play an important role in signals downstream of the pBCR, based on the ability of a constitutively active Ras transgene to phenotypically rescue B cell development to the pre-B cell stage (Shaw et al., 1999a, 1999b). In light of the evidence highlighting the importance of both pBCR- and IL-7R-mediated signaling in B cell development, it is remarkable that a specific mechanism describing their role in this process has yet to be defined.

In a previous report, we presented evidence that cells able to express a functional pBCR exhibit a lowered threshold for proliferation in response to IL-7 (Marshall et al., 1998). We proposed that the interaction of the pBCR and IL-7R pathways results in the selection of pBCR$^+$ cells based on their proliferative advantage over pBCR$^-$ cells in limiting concentrations of IL-7. More recently, another group has demonstrated that inducible expression of the pBCR permits proliferation in low concentrations of IL-7 (Hess et al., 2001). Here, we demonstrate that the ability to proliferate in low IL-7 concentrations requires not only pBCR expression but also a signal downstream of the pBCR. We show that artificially providing a pBCR signal by crosslinking a key component of the pBCR, Igβ, can restore the ability of pBCR-deficient cells to proliferate in low concentrations of IL-7. Additionally, we demonstrate that the ERK/MAPK pathway is required downstream of the pBCR in order to mediate this effect. Furthermore, studies with a panel of IL-7-dependent lines reveal a positive correlation between the expression of the pBCR and constitutive activation of the ERK family MAP kinases. These results reveal a molecular mechanism for selection at this vital checkpoint in B cell development.

Results

Expression of the pBCR Correlates with the Ability to Proliferate in Low Concentrations of IL-7 in Both Fetal and Adult B Cell Precursors

We have proposed a model for selection of pBCR$^+$ bone marrow (BM) cells based on their ability to respond to low levels of IL-7 (Marshall et al., 1998). In this study we designate "high" IL-7 concentrations to be in the range of 1–25 ng/ml, and "low" IL-7 concentrations as 10–100 pg/ml. μMT mice harbor a deletion in the transmembrane domain of the μ Ig heavy chain (HC) gene, resulting in their inability to express surface pBCR complexes (Kitamura et al., 1991). Pro-B cells isolated from μMT adult BM exhibit a significant reduction in proliferation in response to low concentrations of IL-7 relative to

[1] Correspondence: heatherf@oci.utoronto.ca

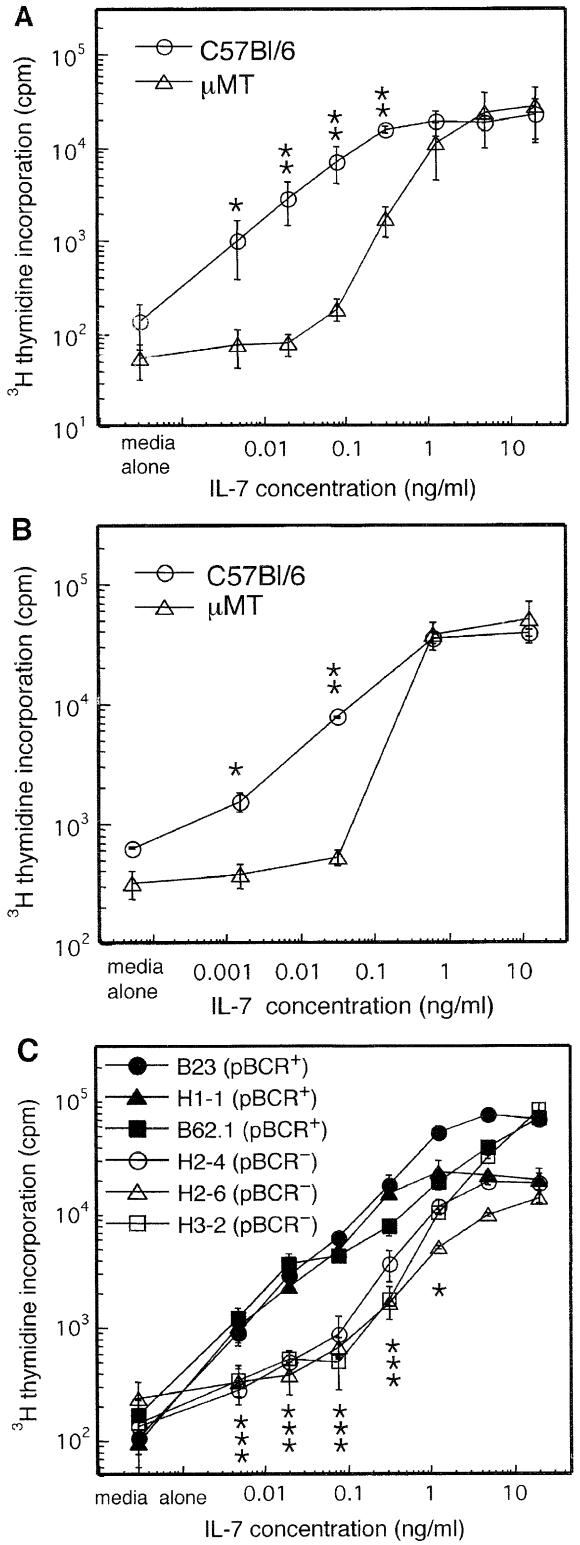

Figure 1. Expression of pBCR Correlates with the Ability to Proliferate in Low Concentrations of IL-7

Proliferation assays were performed in a titration of IL-7 using: (A) pro-B cells sorted from BM of μMT (open triangles) or C57BL/6 (open circles) mice; (B) B cell precursors isolated from d15 FL of μMT (open triangles) or C57BL/6 (open circles) mice; (C) IL-7-dependent cell lines that express the pBCR (B23, closed circles; H1-1, closed triangles; B62.1, closed squares) or do not express the pBCR (H2-4, open circles; H2-6, open triangles; H3-2, open squares). Proliferation was assayed by [^{3}H] thymidine incorporation after 4 days in culture. Data is presented as the mean of triplicate wells; error bars represent SD. Plots are representative of at least four (A), two (B), or five (C) independent experiments. Significance was determined by an unpaired two-tailed student's T test (*** $p < 0.0001$, ** $p < 0.001$, * $p < 0.005$).

WT-derived pro-B cells (Figure 1A; Marshall et al., 1998). As shown in Figure 1B, precursors isolated from the fetal liver environment exhibit a similar response profile, demonstrating that pBCR expression is required for proliferation in response to low concentrations of IL-7 in both developmental compartments. We generated IL-7-dependent cell lines by growing B220$^+$ BM cells at limiting dilutions in the presence of IL-7. Both pBCR$^+$ and pBCR$^-$ lines can be isolated from cultures derived from WT BM. As shown in Figure 1C, populations that express surface pBCR complexes exhibit a 5- to 10-fold increase in proliferation relative to pBCR$^-$ lines in 20–80 pg/ml IL-7. These studies reveal that pBCR surface expression correlates with proliferation in reduced concentrations of IL-7 and provide us with an experimental model system in which biochemical studies may be performed.

Stimulation of the pBCR Signaling Chain on pBCR$^-$ Cells Rescues Proliferation in Low Concentrations of IL-7

The results presented above suggest that a signal resulting from pBCR expression, perhaps derived from the pBCR itself, is required to allow proliferation in low concentrations of IL-7. The HC protein possesses a short cytoplasmic tail, and all signaling by pBCR and BCR complexes is believed to be mediated by the ITAM-containing Igβ and Igα chains. A monoclonal antibody, HM79, which recognizes the extracellular portion of Igβ (Koyama et al., 1997) has been shown to mimic pBCR-derived signals in vivo in a RAG-deficient mouse model (Nagata et al., 1997). We tested the hypothesis that this pBCR-like signal would be sufficient to allow proliferation of *RAG2*$^{-/-}$-derived pBCR$^-$ populations in low concentrations of IL-7 and found, as shown in Figure 2A, that this was the case. Similar results were found utilizing pro-B cells from a μMT background (Figure 2B). These results support our proposal that proliferation in low amounts of IL-7 requires not only pBCR expression but also signals downstream of the pBCR.

Increased ERK/MAPK Activation in the Presence of Combined pBCR and IL-7R Signaling

To examine signals downstream of the pBCR, a panel of IL-7-dependent, pBCR$^+$ cell lines was used to monitor biochemical changes mediated by the IL-7R in the presence or absence of pBCR stimulation. Given that anti-Igβ crosslinking can induce ERK1 activation in RAG-deficient BM cells (Nagata et al., 1997), we tested whether this MAP kinase pathway is involved in pBCR$^+$ cells responding to IL-7. ERK1,2 MAPK activation was measured by performing Western blots with phosphorylation-state specific antibodies to the ERK kinases and also against a target protein of the ERK kinases, p90Rsk (Blenis, 1993). Comparing the time course of ERK and

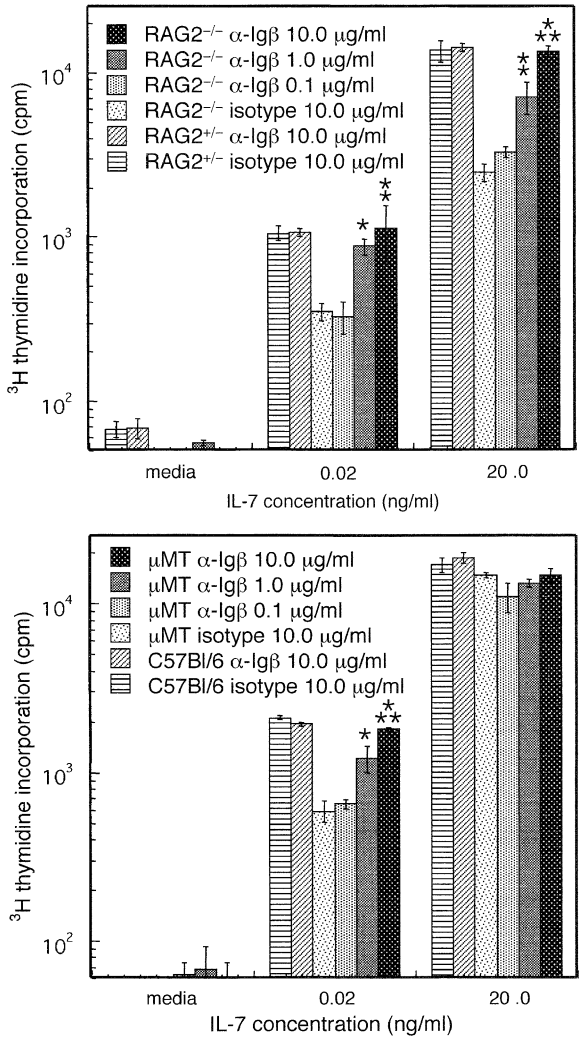

Figure 2. Proliferation of pBCR$^-$ pro-B Cells in Low IL-7 Concentrations Can Be Rescued with anti-Igβ Stimulation

Sorted BM pro-B cells from (A) *RAG2*$^{+/-}$ and *RAG2*$^{-/-}$ and (B) μMT and C57BL/6 mice were plated in triplicate with the indicated concentrations of IL-7. Igβ was crosslinked by the addition of the indicated concentration of the mAb, HM79, at the initiation of culture. Isotype control was added at 10 μg/ml. Proliferation was assayed as in Figure 1. Significance was determined by an unpaired two-tailed student's T test (*** $p < 0.0001$, ** $p < 0.001$, * $p < 0.02$).

Rsk phosphorylation following each independent stimulus reveals that pBCR-derived activation is immediate and transient (Figure 3A, lanes 1, 3, 6, 9, and 12), whereas IL-7-induced phosphorylation is relatively delayed but prolonged (Figure 3A, lanes 1, 2, 5, 8, and 11). As shown in Figure 3A, when both stimuli are combined, more phosphorylated ERK1,2 is detected relative to the level of ERK activation observed in the presence of independent receptor stimulation. In addition, the same result is obtained when a signal through the pBCR is initiated by crosslinking Igβ according to the method described by Nagata et al. (1997) (Figure 3D). These results suggest that activation of the ERK/MAPK pathway can occur downstream of both the pBCR and the IL-7R concurrently. Figure 3B demonstrates that other MAPK path-

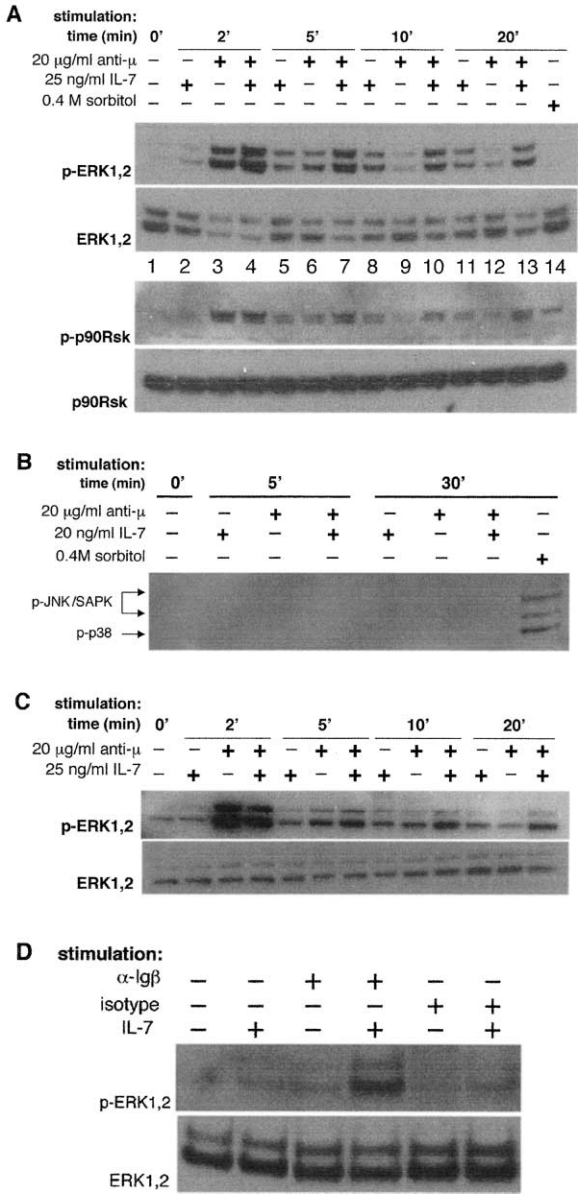

Figure 3. Activation of ERK/MAPK Is Greater Following Combined anti-pBCR and IL-7R Stimulation

(A) The pBCR$^+$ IL-7-dependent line, B62c, was starved of IL-7 and stimulated with anti-μ F(ab')$_2$, IL-7, or both for the indicated times. One sample was treated with 0.4 M sorbitol as a control for p38/JNK stimulation used in (B). Western blotting was performed for phospho-ERK1,2 or phospho-p90Rsk. The blots were stripped and reprobed with a pan-ERK1,2 or a pan-p90Rsk antibody. This blot is representative of at least four experiments and was consistent in two other pBCR$^+$ cell lines.

(B) B62c cells were stimulated as in (A) for the indicated times or with 0.4 M sorbitol for 30 min. Western blotting was performed with a phospho-JNK antibody and then reblotted for phospho-p38, without stripping.

(C) IL-7-responsive precursors were isolated from *RAG2*$^{-/-}$*μHCtg* BM. Cells were stimulated as in (A) and assayed for ERK phosphorylation. This result is representative of two independent experiments.

(D) B62c cells were starved and incubated with biotinylated α-Igβ (HM79) or isotype control for 5 min on ice. Igβ was crosslinked by the addition of streptavidin for an additional 5 min at 37°C in the presence or absence of 20 ng/ml IL-7. Western blotting was performed as in (A). This result is representative of three independent experiments.

ways, p38 and JNK/SAPK, do not appear to be activated in this system.

Increased Activation of ERK Observed in Ex Vivo Isolated pBCR$^+$ Cells following Combined IL-7R and pBCR Stimulation

The results described above were obtained using cell lines that have been selected for continuous in vitro growth in IL-7. To examine the levels of ERK activation following pBCR signals in an ex vivo population, IL-7-dependent cells were grown from *RAG2*$^{-/-}$μ*HCtg* mice (Young et al., 1994). Nonadherent BM cells were grown in IL-7 for 7 days. The phenotype of the resultant population was that of transitional pro-/pre-B cells (99% B220$^+$; 95% CD24$^+$, CD25$^+$; 70% BP1$^+$; 50% CD2$^+$; 40% λ5-SLC$^+$, CD43$^+$; 30% CD22$^+$; not detected: CD40, κLC, CD3, MAC1, GR1; data not shown). Using this culture method, sufficient cell numbers were generated to test whether primary pro-B cells were similar to IL-7-dependent cell lines in their ability to activate ERK. As shown in Figure 3C, additional ERK activation was observed in the presence of both IL-7 and pBCR stimuli relative to the level of ERK activity detected following stimulation of either receptor alone. This confirms that pBCR-derived signals are able to activate ERK when stimulated in the presence of IL-7. The activation of ERK observed in the presence of both stimuli could result from an additive effect of stimulating two independent pools of ERK1,2.

ERK Activation Is Required for Proliferation but Not Differentiation of pBCR$^+$ Cells in Low Concentrations of IL-7

The results obtained by Western blot analysis show that combined IL-7R and pBCR signals lead to higher levels of ERK/MAPK phosphorylation than is found with either stimulus alone. This raises the possibility that the proliferation of pBCR$^+$ cells observed in low concentrations of IL-7 is ERK dependent. According to this hypothesis, the limited amount of ERK activated by low concentrations of IL-7 may be supplemented by ERK activation downstream of the pBCR. In pBCR$^-$ cells, no additional ERK would be phosphorylated, resulting in the failure of cells to proliferate. This model predicts that proliferation in low concentrations of IL-7 is dependent on reaching a threshold of ERK phosphorylation. To test this prediction, an inhibitor of MEK1 kinase (Alessi et al., 1995) was used to specifically inhibit the activation of ERK/MAPK (Figure 4A) by pBCR$^+$ and pBCR$^-$ cells in a titration of IL-7. The proliferation of pBCR$^+$ populations (Figure 4B) but not a pBCR$^-$ line (Figure 4C) is inhibited in a dose-dependent manner in the presence of the MEK1 inhibitor, PD98059. In contrast, treatment with LY294002, a PI3K inhibitor (Vlahos et al., 1994), prevented proliferation in both pBCR$^+$ and pBCR$^-$ cells at all concentrations of IL-7, consistent with the known requirement for PI3K in IL-7-induced mitogenic responses (Corcoran et al., 1996) (Figures 4B and 4C). This result was extended by examining IL-7-induced proliferation of freshly sorted pro-B cells from *RAG2*$^{+/-}$ and *RAG2*$^{-/-}$ mice in the presence of another inhibitor of MEK1/2, UO126 (Favata et al., 1998). As observed in the IL-7-dependent lines, MEK inhibition leads to a loss of proliferation of RAG$^+$ pro-B cells (Figure 5A). This

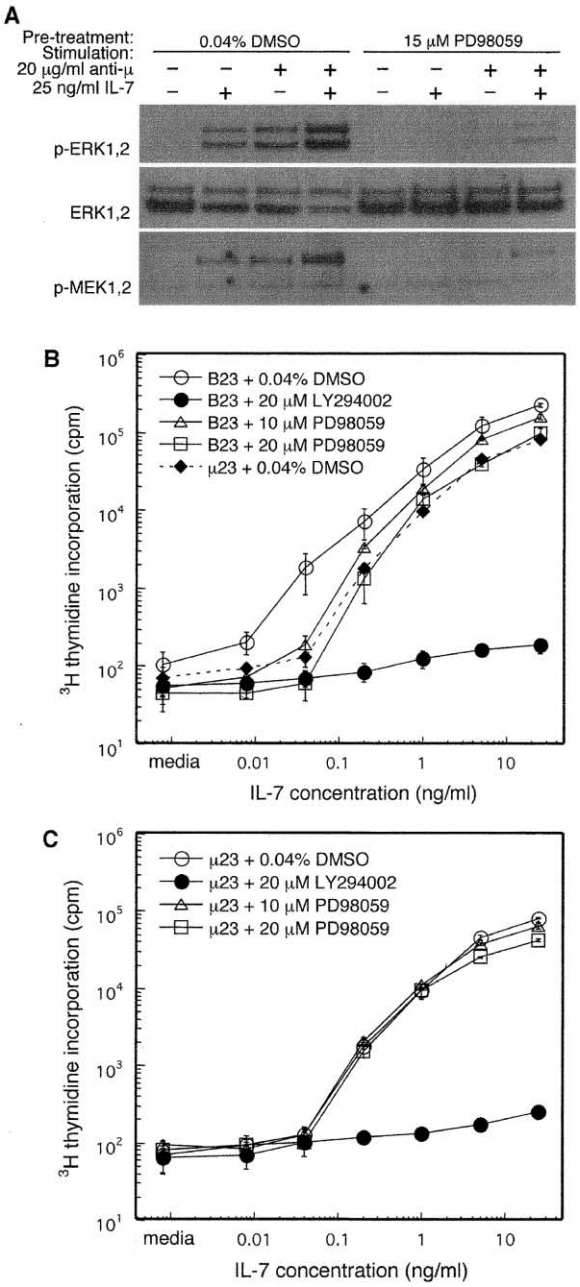

Figure 4. Inhibition of MEK Kinase Prohibits Proliferation of IL-7-Dependent pBCR$^+$ Cells in Low Concentrations of IL-7

(A) B62c cells were starved and incubated in the presence of DMSO or a MEK inhibitor, PD98059, for 1 hr prior to stimulation. Western blotting for phosphorylated MEK1,2 and ERK1,2 reveals a loss of MEK activity and ERK activation following treatment with this inhibitor.

(B and C) Proliferation in a titration of IL-7 was assayed in the presence of PD98059, at 10 μM (open triangles) and 20 μM (open squares) or in the presence of solvent alone (DMSO, open circles). Populations tested include (B) B23, a pBCR$^+$ IL-7-dependent line, and (C) μ23, an IL-7-dependent, pBCR$^-$ line. The proliferation of μ23 (dashed line) is included in (B) as a reference for proliferation expected in the absence of pBCR-derived signals. Treatment of both populations with a PI3K inhibitor, LY294002 (closed circles), inhibits proliferation at all IL-7 concentrations. Data is presented as the mean of triplicate wells, and error bars represent SD. Results are representative of at least three independent experiments and are consistent in multiple IL-7-dependent cell lines.

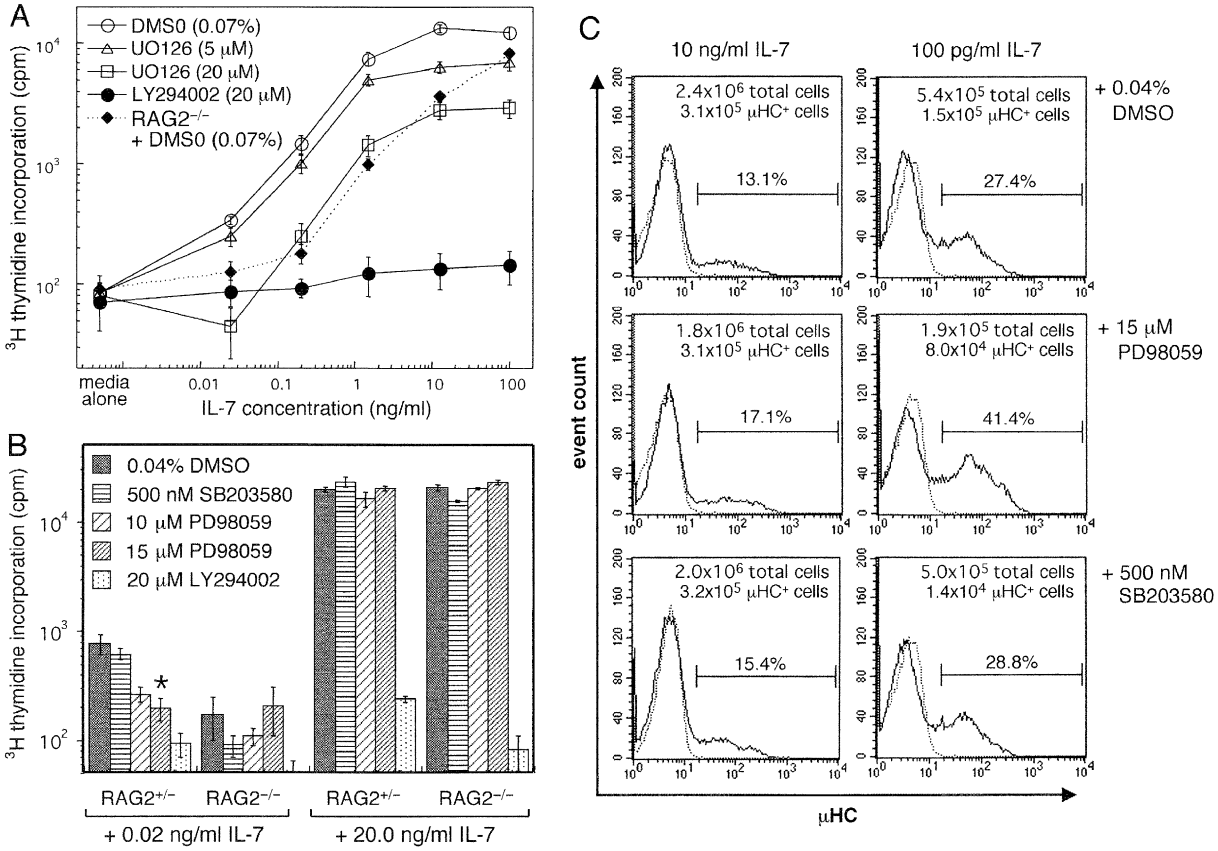

Figure 5. ERK1,2 Activation Is Required for the Proliferation but Not Differentiation of Sorted pro-B Cells in Low Concentrations of IL-7

(A) Proliferation of sorted pro-B cells from *RAG2*$^{+/-}$ mice was assayed in a titration of IL-7 in the presence of another MEK inhibitor, U0126, at 5 μM (open triangles) or 20 μM (open squares), the PI3K inhibitor (LY294002, closed circles), or solvent alone (open circles). The proliferation of untreated *RAG2*$^{-/-}$ cells is included for reference (dashed line).

(B) RAG2$^{+/-}$ and *RAG2*$^{-/-}$ pro-B cells were cultured in high or low concentrations of IL-7 in the presence of a MEK inhibitor (PD98059), p38 inhibitor (SB203580), PI3K inhibitor (LY294002), or DMSO control. Proliferation was assayed by [^{3}H] thymidine incorporation on day 4 of culture. Error bars represent SD of triplicate wells. Significance was determined by an unpaired two-tailed student's T test (*$p < 0.005$).

(C) FACS analysis of μHC expression was performed in sorted *RAG2*$^{+/-}$ pro-B cells grown for 5 days in the presence of the indicated concentrations of IL-7 and inhibitor, or DMSO control. The absolute number of total and μHC$^+$ cells recovered in each condition is indicated. Results shown are representative of two (A and C) or four (B) independent experiments.

effect is consistent when PD98059 is used to inhibit the RAG2$^+$ precursors in low concentrations of IL-7 (Figure 5B). Only *RAG2*$^{+/-}$ cells exhibit dose-dependent inhibition of proliferation when cultured with the MEK inhibitor. In contrast, neither *RAG2*$^{+/-}$ nor *RAG2*$^{-/-}$ populations are inhibited by treatment with a p38/MAP kinase inhibitor (Cuenda et al., 1995), suggesting a specific involvement of the ERK/MAP kinase pathway (Figure 5B). Finally, the sorted pro-B cells exhibit sensitivity to PI3K inhibition at all concentrations of IL-7 (Figure 5B).

FACS analysis of the sorted pro-B cells was performed to examine the possibility that treatment with the MEK inhibitors prevented their proliferation by blocking maturation to the pre-B cell stage. As shown in Figure 5C, inhibition of ERK activation by PD98059 treatment does not prevent the development of μHC$^+$ cells in low concentrations of IL-7. In fact, there is an increase in the percentage of mature cells in the MEK1-inhibited population relative to that observed following treatment with solvent alone or with the p38 inhibitor. Indeed, while a reduced absolute number of cells is recovered from PD98059-treated cultures grown in low amounts of IL-7 (2.7 ± 0.2-fold reduction; Figure 5C and data not shown), a less dramatic reduction in μHC$^+$ cell number is observed (1.8 ± 0.05-fold reduction; Figure 5C and data not shown) in conditions that elicit very low levels of proliferation (Figures 5A and 5B). Together, these results suggest that pBCR-dependent activation of ERK is required for proliferation but not differentiation in low concentrations of IL-7.

Enforced Survival Is Insufficient to Permit pBCR$^-$ Populations to Proliferate in Low Concentrations of IL-7

The result that ERK activity is required for proliferation in low concentrations of IL-7 does not distinguish between a direct mitogenic effect of ERK downstream targets and the possibility that induced survival signals permit an alternative proliferative stimulus to act (Nagaoka et al., 2000; Shaw et al., 1999b). To address the

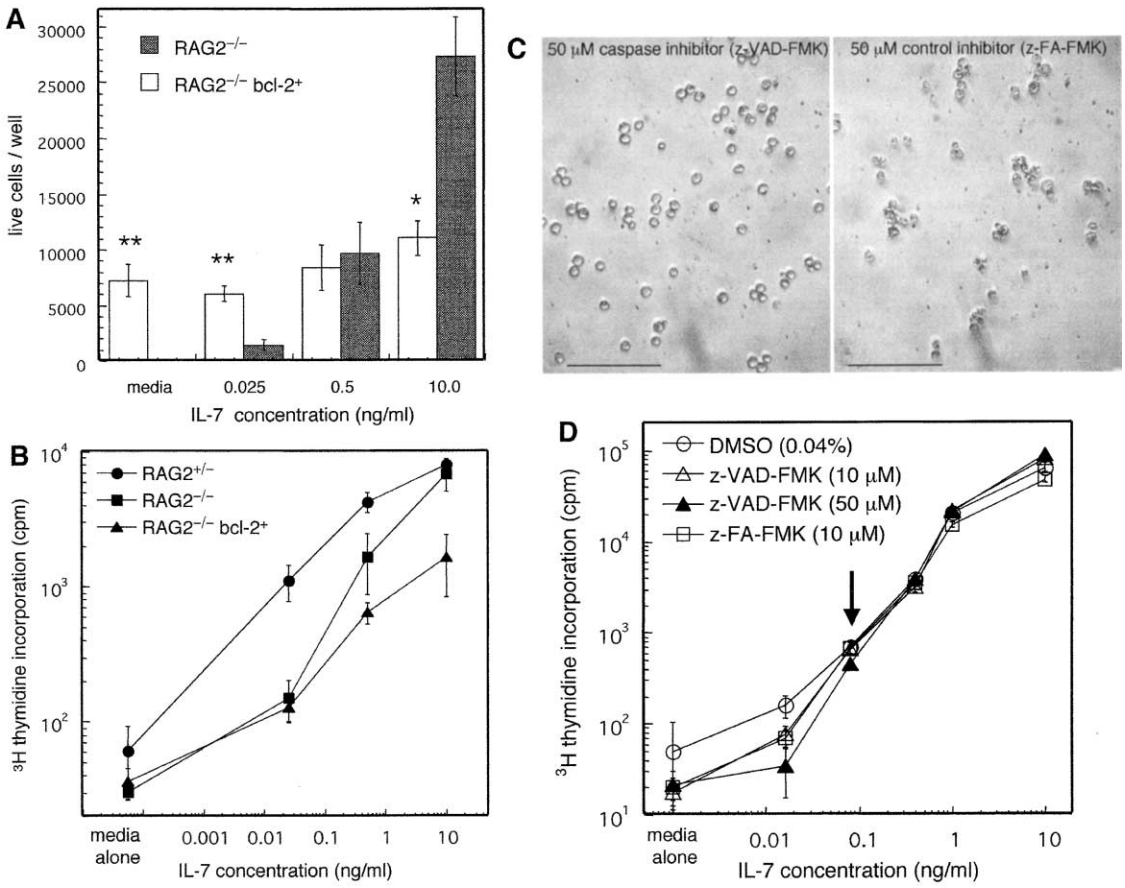

Figure 6. Enforced Survival of pBCR$^-$ Cells Is Insufficient to Allow Their Proliferation in Low Concentrations of IL-7

(A and B) Pro-B cells were sorted from *RAG2*$^{+/-}$ and *RAG2*$^{-/-}$ mice with or without a *bcl-2* transgene, plated at 5000 cells/well in a titration of IL-7, and cultured for 4 days. Replicate triplicate wells were assayed for total cell recovery (A) or proliferation (B). Results shown are representative of two independent experiments, and error bars represent SD. Significance was determined by an unpaired two-tailed student's T test (** $p < 0.001$, * $p < 0.002$).

(C and D) H3-3, a pBCR$^-$ IL-7-dependent cell line, was grown in a titration of IL-7 in the presence of a caspase inhibitor (z-VAD-FMK, [C] left panel, [D] triangles) or a control cathepsin B inhibitor (z-FA-FMK, [C] right panel, [D] squares) for 4 days. Immediately prior to the addition of [^{3}H] thymidine to assay proliferation (D), micrographs were taken of wells grown in 100 pg/ml IL-7 (indicated with an arrow in [D]) to assess cell survival. Representative pictures are shown in (C). The size bar indicates 50 μM. Results shown are representative of two independent experiments and are consistent in multiple cell lines.

question of whether survival is sufficient to allow proliferation of pBCR$^-$ cells in low IL-7, we sorted pro-B cells from *RAG2*$^{-/-}$*bcl-2* transgenic mice and compared their ability to proliferate with that of *RAG2*$^{-/-}$- and *RAG2*$^{+/-}$-derived populations. As shown in Figure 6A, while expression of the *bcl-2* transgene does permit the survival of *RAG2*$^{-/-}$ cells as the concentration of IL-7 is lowered in culture (Figure 6A), it is insufficient to allow proliferation in low concentrations of IL-7 (Figure 6B). We recognize that overexpression of *bcl-2* may prevent proliferation by altering cell cycle state (Figure 6B; Mazel et al., 1996; Vairo et al., 1996). Therefore, we also treated a pBCR$^-$ IL-7-dependent cell line with a wide-spectrum caspase inhibitor (z-VAD-FMK, Thornberry and Lazebnik, 1998) or a control inhibitor (Z-FA-FMK, Schotte et al., 1999). While inhibition of caspase activity does promote survival in low concentrations of IL-7 (Figure 6C), this method of enforced survival is not sufficient to allow pBCR$^-$ cells to proliferate (Figure 6D).

Higher Constitutive Levels of ERK Activation in pBCR$^+$ IL-7-Dependent Lines Relative to pBCR$^-$ Lines

The model described above suggests that pBCR expression leads to proliferation in reduced concentrations of IL-7 by activating ERK. We examined a panel of unstimulated IL-7-dependent cell lines to determine whether there was a correlation between pBCR expression and constitutive levels of ERK/MAPK activation. As shown in Figure 7A, elevated levels of constitutively phosphorylated ERK1,2 are observed in pBCR$^+$ lines (lanes 1, 2, and 3) relative to pBCR$^-$ lines (lanes 4, 5, and 6). Furthermore, a pBCR$^+$ cell line selected for its ability to grow in the absence of IL-7 exhibits the highest background levels of ERK phosphorylation (Figure 7A, lane 1: IND). Also, while this line retains its ability to proliferate in response to IL-7 stimulation (Figure 7B), short-term treatment with IL-7 does not increase ERK1,2 phosphorylation (Figure 7A, lane 1 versus 7) in contrast to the IL-7-dependent lines (Figure 7A, lanes 2–6 versus

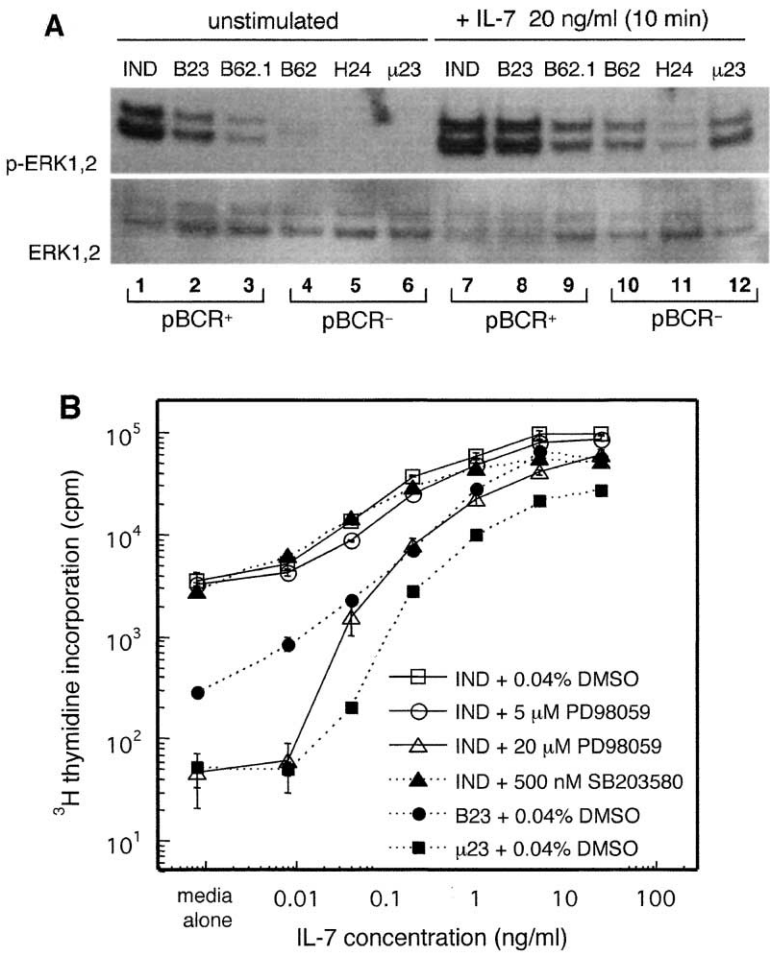

Figure 7. Constitutive Levels of ERK Phosphorylation Correlate with pBCR Expression and the Ability to Proliferate in Low IL-7

(A) A panel of cell lines that express (IND, B23, B62.1) or do not express (B62, H24, μ23) the pBCR were starved of IL-7 and left unstimulated (lanes 1–6) or stimulated with IL-7. Lysates were assayed for phosphorylated ERK1,2 as in Figure 3.

(B) The IND line (lanes 1 and 7) was assayed for proliferation in response to an IL-7 titration in the presence of 5 μM PD98059 (open circles), 20 μM PD98059 (open triangles), 500 nM SB203580 (p38 inhibitor, closed triangles), or solvent alone (DMSO, open squares). The proliferation of uninhibited pBCR$^+$ (B23, closed circles) and pBCR$^-$ (μ23, closed squares) cells are included as reference IL-7-dependent lines.

lanes 8–12). Finally, the IND line is no longer able to proliferate in the absence of IL-7 when treated with the MEK1 inhibitor but is unaffected by the presence of the p38 inhibitor, SB203580 (Figure 7B and data not shown). These results are consistent with our model that pBCR expression leads to ERK activation, which promotes selection by permitting proliferation in concentrations of IL-7 that are insufficient to support the expansion of pBCR$^-$ cells.

Discussion

The generation of a functionally rearranged μHC that can associate with the component chains of the pBCR is an absolute requirement for B cell development. To date, the mechanism underlying this requirement is largely unexplained (Meffre et al., 2000). In this report, we show that (1) pBCR expression by adult and fetal pre-B cells and IL-7-dependent cell lines correlates with the ability to proliferate in low concentrations of IL-7; (2) a signal downstream of the pBCR signaling chain is sufficient to mediate this effect; (3) concurrent stimulation of the pBCR and the IL-7R specifically activates the ERK/MAP kinase pathway; (4) ERK activation is required for pBCR-mediated proliferation but not differentiation in minimal IL-7; and (5) expression of the pBCR in IL-7-dependent cell lines correlates with increased constitutive ERK phosphorylation. Together, these observations suggest a simple molecular mechanism that mediates the pro-B to pre-B transition checkpoint. We show that the levels of phosphorylated ERK produced in response to IL-7 stimulation is increased in cells receiving a pBCR signal. We suggest that this additional source of activated kinase, unavailable to pBCR$^-$ cells, creates sufficient downstream effectors to allow the proliferation of the pBCR$^+$ population in low concentrations of IL-7.

We have shown here that a signal downstream of the pBCR signaling chain is sufficient to alter the IL-7 responsiveness of a developmentally arrested population (see Figure 2). Cellular changes following the membrane insertion of the pBCR include allelic exclusion at the HC locus, a limited burst of cell division, a variety of cell surface marker changes, and eventual initiation of recombination at the light chain locus (Meffre et al., 2000). The components of the pBCR complex have been manipulated in order to dissect which portion signals for which changes within the cell. In most model systems, the ITAM sequences of Igα or Igβ appear to be required to mediate pBCR-derived effects (Meffre et al., 2000). However, a transgenic model using a μHC mutated such that it could not associate with Igα/β was used to show that development was blocked, while allelic exclusion remained intact (Cronin et al., 1998). Simi-

larly, transgenic LMP2A models indicate that expression of an ITAM is sufficient to turn off recombination at the HC locus (Caldwell et al., 1998; Merchant et al., 2000). In addition, the treatment of μMT pro-B cells with an anti-Igβ monoclonal antibody can signal allelic exclusion in the absence of the pBCR (Maki et al., 2000). These results demonstrate that activation of an ITAM can mediate allelic exclusion prior to formation of the pBCR complex and may indicate that Igβ signals have a role in differentiation prior to those downstream of the pBCR. This raises an interesting point regarding our own use of the anti-Igβ antibody to mimic a pBCR signal, in that a "pro-BCR" signal induced by HM79 may be sufficient to mediate a decrease in the IL-7 response threshold. However, Iritani et al. (1999) have demonstrated that pBCR-like signals mediated by a constitutively activate cRaf-1 transgene are distinct from those that lead to allelic exclusion, suggesting that the proliferation we induce with anti-Igβ treatment may not be due to the early pro-BCR signaling capacity of the Igβ ITAM. In either case, we have shown that Igβ signals can mediate a decrease in the IL-7 response threshold and that a functional μHC is required in the absence of artificially stimulated Igβ.

We show that activation of the ERK/MAP kinase pathway is required to permit proliferation of pBCR$^+$ cells in low concentrations of IL-7. Other groups have shown that ERK can be activated downstream of the pBCR, BCR, and the IL-7R (Healy et al., 1997; Nagata et al., 1997; Sutherland et al., 1996; Zeng et al., 1994). We have demonstrated that pBCR expression correlates with an increase in constitutively phosphorylated ERK (Figure 7A) and that in the absence of ERK activation, pBCR$^+$ lines exhibit proliferation equivalent to pBCR$^-$ lines (Figures 4B and 5A). However, we cannot conclude from these results that phosphorylated ERK leads directly to proliferation. We considered that pBCR expression could transmit a survival signal, allowing cells to persist and proliferate in cultures containing low amounts of IL-7. This is supported by the observations that constitutive activation of the Ras pathway mediates survival in developing B cells (Nagaoka et al., 2000; Shaw et al., 1999b) and that transgenic expression of the LMP2A ITAM confers survival signals to pBCR$^-$ cells, even in the absence of phenotypic differentiation (Caldwell et al., 2000). Consistent with the observation that *bcl-2* transgene expression does not rescue development in *RAG2*$^{-/-}$ mice (Young et al., 1997), we demonstrated that simply keeping pBCR$^-$ cells alive is not sufficient to permit proliferation in the presence of low IL-7 concentrations (Figure 6). These results do not preclude a role for survival signals mediated by either the pBCR and/or the IL-7R, but it is clear that more than the survival of pBCR$^+$ cells accounts for their ability to proliferate in low concentrations of IL-7. Alternatively, pBCR-derived signals may induce maturational changes that lead to ERK-dependent proliferation. We have observed that ERK inhibition does not prevent phenotypic maturation in our IL-7 cultures (Figure 5C). In fact, considering the absolute number of μHC$^+$ cells recovered from wells that exhibit very little thymidine incorporation (Figure 5), the frequency of maturation may be enhanced in low concentrations of IL-7 in the absence of ERK activity. While we cannot rule out that a series of maturation events leads to the proliferation of pBCR$^+$ cells in low concentrations of IL-7, it appears that the requirement for ERK activation in the lowering of the IL-7 response threshold is downstream of pBCR expression.

As discussed above, activated Ras transgenes can rescue development in pBCR-deficient animals. While this effect may be due to multiple arms of the MAP kinase pathway, we believe that our observations are specific to the ERK1,2 family members. No phosphorylation of the JNK/SAPK and p38 proteins is observed by Western blot (Figure 3B), and an inhibitor of p38 activity is unable to alter the proliferation of any IL-7-dependent line at any IL-7 concentration tested (Figures 5B and 7B; data not shown). No data concerning the effects of ERK1 deficiency on the B cell lineage are available, and the alterations observed in the T lineage are minimal, likely due to some amount of functional redundancy between the two proteins (Pages et al., 1999). The types of stimuli that lead to either proliferation or differentiation via ERK activation in nerve cells have been studied extensively (Fukunaga and Miyamoto, 1998). Intriguingly, the time course of ERK activation may alter the outcome of the signal by determining whether or not activated ERK translocates to the nucleus (Marshall, 1995). Preliminary studies suggest that phosphorylated ERK does translocate to the nucleus following all of stimuli used in this study (data not shown), but further experiments are in progress to address whether a pBCR signal can alter the subcellular localization of activated ERK.

Considerable speculation and effort have surrounded the attempt to identify the natural ligand for the pBCR. In the absence of an identified ligand, it has been postulated that signaling through the pBCR is initiated simply by surface expression of the receptor (Kurosaki, 2000). According to our model, this would suggest that pBCR$^+$ cells should express constitutively higher levels of activated ERK. This is, in fact, what is observed in our panel of cell lines (Figure 7A). The parallel observation has been made in the T cell lineage, where ERK phosphorylation was induced following pTCR expression, in the absence of any exogenous stimulation (Michie et al., 1999). Indeed, in our in vitro experiments, the mere presence of surface-expressed pBCR is sufficient for proliferation in low concentrations of IL-7 (Marshall et al., 1998) (Figure 1). In addition, this proliferation is unaltered by the addition of antibodies that crosslink the pBCR (Stoddart et al., 2001) (Figure 2; data not shown). However, while no known ligand has been added to these cultures, it is possible that a ligand for the pBCR may exist on the cultured cells themselves.

The results presented here and discussed above support our model for selection of pBCR$^+$ cells, in which a signal downstream of the pBCR induces ERK/MAP kinase phosphorylation to a level that is sufficient to allow proliferation in low concentrations of IL-7. The absence of pBCR-derived ERK activation in pBCR$^-$ cells would prevent them from proliferating at concentrations that support pBCR$^+$ cell growth. This sets up a situation in which pBCR$^+$ cells will be selectively expanded in an environment where IL-7 is limiting. Apparently, these conditions still allow Ig$^+$ cells to arise in the nonexpanding population, offering the possibility of recovery should an appropriate receptor be expressed. Accurate

in vivo measurement of IL-7 concentrations is difficult and may not reflect the amount of IL-7 that is functionally active and available to the B cell precursors. Extracellular matrix proteins can bind and inactivate cytokines (Amara et al., 1999; Clarke et al., 1995), while cell surface heparan sulfate on B cells and stromal cells can specifically bind active IL-7 (Borghesi et al., 1999). Another report has shown that a stromal-derived protein can dimerize IL-7 monomers and that exposure to these complexes is required to induce IL-7-monomer responsiveness at the pro-B cell stage (Lai et al., 1998; McKenna et al., 1998). Finally, the ability of individual stromal cells to produce IL-7 varies substantially (Gimble et al., 1989; Kincade et al., 1989; Stephan et al., 1998). Together, these observations suggest that the availability of IL-7 in the mouse may be quite dynamic. Several groups have assayed the in vivo effects of IL-7 by artificially increasing the concentration by way of enforced transgenic expression, systemic injection, or continuous subcutaneous infusion (Mertsching et al., 1996; Valenzona et al., 1998, 1996). All of these methods have revealed excessive proliferation of both pro- and pre-B cell populations, suggesting that the in vivo concentrations were limiting under unmanipulated conditions. A report has been published describing the effects of IL-7 transgene expression on B cell development in mice with specific defects in this process (Ceredig et al., 1999). They demonstrate that increased in vivo IL-7 concentrations do not restore development in pBCR-deficient backgrounds but can allow populations of pBCR$^-$ cells to exist in the periphery. This is in agreement with our model that increased responsiveness to IL-7 plays a selective role in normal B cell development, ensuring that only pBCR$^+$ cells are able to continue to mature and emigrate to the periphery. It should be noted that while IL-7 can induce proliferation and other effects in human B cell precursors, there is no apparent requirement for an IL-7 signal during human B cell development (LeBien, 2000). We would propose that an alternative factor is responsible for selective expansion of pBCR$^+$ precursors in the human bone marrow environment. In summary, our data provide a rational basis for understanding the molecular processes that regulate this vital checkpoint in B cell development.

Experimental Procedures

Mice

μMT mice (Kitamura et al., 1991) were obtained through Dr. L. Shultz (The Jackson Laboratory, Bar Harbor, ME). *RAG2*$^{-/-}$ (Shinkai et al., 1992) and *RAG2*$^{-/-}$ HC186 transgenic (Young et al., 1994) mice were provided by Dr. F. Alt (The Children's Hospital, Boston, MA). *RAG2*$^{+/-}$ mice were generated by crossing *RAG2*$^{-/-}$ mice with C57BL/6 mice (Jackson). *bcl-2* transgenic mice (Jackson) were crossed with *RAG2*$^{-/-}$ mice to obtain double mutants. All mice were bred and maintained at the Ontario Cancer Institute animal facility (Toronto, Canada) and were used between 6 and 10 weeks of age. Timed pregnancies of C57BL/6 and μMT mice were established by mating mice overnight and observing vaginal plugs in the morning, day 0.

Cell Purification

Cell suspensions of BM and day 15 FL were prepared as described (Marshall et al., 1998; Ray et al., 1998). B cell progenitors (CD19$^+$, CD43$^+$, μHC$^-$) were enriched from BM as described (Marshall et al., 1998) with a "MoFlo" (Cytomation, Inc.) high speed cell sorter equipped with a dual-beam, water-cooled Innova Enterprise II Argon ion laser regulated to emit 125 mW power at 488 nm. CyCLOPS Summit software builds b574 and b619 were used for acquisition and analysis. Typically, 0.5%–1.0% of total BM was recovered from C57BL/6 and *RAG2*$^{+/-}$ mice, and 3%–5% of μMT and *RAG2*$^{-/-}$ BM was recovered from the sort. Sorted populations were routinely >97% pure. Enrichment of pro-B cells from d15 FL was performed by panning as described (Ray et al., 1998). Typically, 2–3 × 10^7 cells were recovered per d15 C57BL/6 or μMT FL, ~0.8%–1% of which were B220$^+$.

Cell Culture Conditions

Cells were grown in OptiMEM (Gibco) supplemented with 10% FCS, 50 μM 2-ME, 2.4 g/L $NaHCO_3$, 100 μg/ml penicillin, 100 μg/ml streptomycin, and the indicated factors. For proliferation assays, cells were plated in triplicate at 5000 cells/well in 96-well flat-bottom plates in the presence of the indicated concentration of recombinant mouse IL-7 (R&D Systems, Minneapolis, MN) or medium alone. Anti-Igβ (HM79, type 2 hamster IgG, Pharmingen) was added at the indicated concentrations at the initiation of culture. Inhibitors (PD98059, U0126, LY294002 [New England Biolabs]; SB203580, z-VAD-FMK, z-FA-FMK [Calbiochem]) were solubilized in DMSO and added at the initiation of culture. Proliferation of cells was measured on day 4 by addition of 0.5 μCi [^{3}H] thymidine (Mandel, Guelph, Ontario) 6 hr prior to the end of culture. Lysed cells were harvested onto microplate filters, and radioactivity was measured in a scintillation counter (Topcount System, Canberra Packard, Meriden, CT). For FACS analysis of differentiation shown in Figure 5C, sorted pro-B cells were plated at 2.5 × 10^4 cells/well in a 24-well plate, with the indicated concentrations of IL-7 and inhibitors. FACS analysis was performed as described (Marshall et al., 1998) with a μHC-specific monoclonal antibody, 33.60-FITC. Cell counts shown in Figure 6A were performed in duplicate on triplicate wells that had been set up in parallel with wells assayed for proliferation. IL-7-dependent lines were maintained in OptiMEM with 5% FCS and 5–10 ng/ml murine IL-7 from the supernatant of the stably transfected J558 line (Dr. A. Cumano, Pasteur Institute, Paris). IL-7-responsive pBCR$^+$ populations were isolated from *RAG2*$^{-/-}$*μHCtg* BM by selection of nonadherent cells grown in OptiMEM + 10% FCS and 10 ng/ml murine IL-7. IL-7-dependent cell lines were isolated by limiting dilution of B220$^+$-panned BM cells (Ray et al., 1998) grown in the absence of stromal cells in OptiMEM + 10% FCS + ~5 ng/ml murine IL-7 for 6 to 8 weeks. The IL-7-independent cell line, IND, was isolated by limiting dilution of the pBCR$^+$ line, B23, grown in ~20 pg/ml IL-7 for 6 weeks.

Cell Stimulation and Western Blotting

Prior to stimulation, cells were washed in OptiMEM + 0.5% FCS to remove IL-7 and starved for 1.5–2 hr in a 37°C humidified incubator at 5 × 10^6 cells/ml. Cells were resuspended at 10^7 cells/ml at 37°C and stimulated by the addition of 10–20 μg/ml F(ab')$_2$ goat anti-mouse μHC (Jackson Immunoresearch), 10–25 ng/ml murine IL-7, or both, for the times indicated. Positive control samples for JNK/p38 activation were stimulated with 0.4 M sorbitol (Sigma) for 30 min. Cells were lysed at 5 × 10^7 cells/ml in 1% NP40, 150 mM NaCl, 20 mM Tris-HCl (pH 7.4), 1 mM EDTA, 10 mM sodium fluoride, 1 mM sodium orthovanadate, 5 mM sodium pyrophosphate, 1 mM PMSF, and 5 μg/ml aprotinin and leupeptin (Boehringer Mannheim) on ice for 20 min. The detergent insoluble fraction was removed by centrifugation. Protein samples (equivalent of 6 × 10^5–1.2 × 10^6 cells) were mixed with 4× NuPAGE sample buffer (Invitrogen) and 0.7 M 2-ME, resolved on a gradient NuPAGE gel, and transferred to a PVDF membrane in 20 mM Tris/150 mM glycine/20% methanol. Detection of phosphorylated ERK1,2, p90Rsk, MEK1,2, JNK/SAPK, and p38 was performed according to the manufacturer's instructions (NEB). For loading controls, membranes were stripped in 6.25 mM TRIS (pH 6.8), 2% SDS, and 100 mM 2-ME (50°C, 25 min), washed in TBST, and reprobed as above. Stimulation by Igβ cross-linking was adapted from the method of Nagata et al. (1997). Cells were starved as above and incubated at 2 × 10^7 cells/ml on ice with 30 μg/ml HM79-biotin or isotype control for 5 min. Samples were stimulated for a further 5 min at 37°C with the addition of 20 μg/ml streptavidin with or without 20 ng/ml IL-7. The reaction was stopped by the addition of cold wash buffer (150 mM NaCl, 20 mM Tris-

HCl [pH 7.4], 1 mM EDTA, 10 mM sodium fluoride, 1 mM sodium orthovanadate, and 5 mM sodium pyrophosphate) followed by centifugation. Cell lysis and sample preparation were performed as above.

Acknowledgments

This work has been supported by the CIHR and the NCIC. H.E.F. is funded by a CIHR Doctoral Research Award. We are grateful to Drs. K. Rajewsky and F. Alt for providing mutant mice. We thank C. Cantin for his expert assistance with cell sorting and flow cytometry. We thank C. Milne and S. Lax for critical reading of this manuscript and members of the Paige lab for engaging discussions.

Received March 22, 2001; revised August 24, 2001.

References

Alessi, D.R., Cuenda, A., Cohen, P., Dudley, D.T., and Saltiel, A.R. (1995). PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase in vitro and in vivo. J. Biol. Chem. *270*, 27489–27494.

Amara, A., Lorthioir, O., Valenzuela, A., Magerus, A., Thelen, M., Montes, M., Virelizier, J.L., Delepierre, M., Baleux, F., Lortat-Jacob, H., and Arenzana-Seisdedos, F. (1999). Stromal cell-derived factor-1alpha associates with heparan sulfates through the first beta-strand of the chemokine. J. Biol. Chem. *274*, 23916–23925.

Benbernou, N., Muegge, K., and Durum, S.K. (2000). Interleukin (IL)-7 induces rapid activation of Pyk2, which is bound to Janus kinase 1 and IL-7Rα. J. Biol. Chem. *275*, 7060–7065.

Blenis, J. (1993). Signal transduction via the MAP kinases: proceed at your own RSK. Proc. Natl. Acad. Sci. USA *90*, 5889–5892.

Borghesi, L.A., Yamashita, Y., and Kincade, P.W. (1999). Heparan sulfate proteoglycans mediate interleukin-7-dependent B lymphopoiesis. Blood *93*, 140–148.

Caldwell, R.G., Wilson, J.B., Anderson, S.J., and Longnecker, R. (1998). Epstein-Barr virus LMP2A drives B cell development and survival in the absence of normal B cell receptor signals. Immunity *9*, 405–411.

Caldwell, R.G., Brown, R.C., and Longnecker, R. (2000). Epstein-Barr virus LMP2A-induced B-cell survival in two unique classes of EmuLMP2A transgenic mice. J. Virol. *74*, 1101–1113.

Ceredig, R., Andersson, J., Melchers, F., and Rolink, A. (1999). Effect of deregulated IL-7 transgene expression on B lymphocyte development in mice expressing mutated pre-B cell receptors. Eur. J. Immunol. *29*, 2797–2807.

Clarke, D., Katoh, O., Gibbs, R.V., Griffiths, S.D., and Gordon, M.Y. (1995). Interaction of interleukin 7 (IL-7) with glycosaminoglycans and its biological relevance. Cytokine *7*, 325–330.

Corcoran, A.E., Smart, F.M., Cowling, R.J., Crompton, T., Owen, M.J., and Venkitaraman, A.R. (1996). The interleukin-7 receptor α chain transmits distinct signals for proliferation and differentiation during B lymphopoiesis. EMBO J. *15*, 1924–1943.

Cronin, F.E., Jiang, M., Abbas, A.K., and Grupp, S.A. (1998). Role of mu heavy chain in B cell development. I. Blocked B cell maturation but complete allelic exclusion in the absence of Ig alpha/beta. J. Immunol. *161*, 252–259.

Cuenda, A., Rouse, J., Doza, Y.N., Meier, R., Cohen, P., Gallagher, T.F., Young, P.R., and Lee, J.C. (1995). SB 203580 is a specific inhibitor of a MAP kinase homologue which is stimulated by cellular stresses and interleukin-1. FEBS Lett. *364*, 229–233.

Cumano, A., Dorshkind, K., Gillis, S., and Paige, C.J. (1990). The influence of S17 stromal cells and interleukin 7 on B cell development. Eur. J. Immunol. *20*, 2183–2189.

Della Rocca, G.J., van Biesen, T., Daaka, Y., Luttrell, D.K., Luttrell, L.M., and Lefkowitz, R.J. (1997). Ras-dependent mitogen-activated protein kinase activation by G protein-coupled receptors. Convergence of Gi- and Gq-mediated pathways on calcium/calmodulin, Pyk2, and Src kinase. J. Biol. Chem. *272*, 19125–19132.

Favata, M.F., Horiuchi, K.Y., Manos, E.J., Daulerio, A.J., Stradley, D.A., Feeser, W.S., Van Dyk, D.E., Pitts, W.J., Earl, R.A., Hobbs, F., et al. (1998). Identification of a novel inhibitor of mitogen-activated protein kinase kinase. J. Biol. Chem. *273*, 18623–18632.

Fukunaga, K., and Miyamoto, E. (1998). Role of MAP kinase in neurons. Mol. Neurobiol. *16*, 79–95.

Gimble, J.M., Pietrangeli, C., Henley, A., Dorheim, M.A., Silver, J., Namen, A., Takeichi, M., Goridis, C., and Kincade, P.W. (1989). Characterization of murine bone marrow and spleen-derived stromal cells: analysis of leukocyte marker and growth factor mRNA transcript levels. Blood *74*, 303–311.

Healy, J.I., Dolmetsch, R.E., Timmerman, L.A., Cyster, J.G., Thomas, M.L., Crabtree, G.R., Lewis, R.S., and Goodnow, C.C. (1997). Different nuclear signals are activated by the B cell receptor during positive versus negative signaling. Immunity *6*, 419–428.

Hess, J., Werner, A., Wirth, T., Melchers, F., Jack, H.-M., and Winkler, T.H. (2001). Induction of pre-B cell proliferation after de novo synthesis of the pre-B cell receptor. Proc. Natl. Acad. Sci. USA *98*, 1745–1750.

Hofmeister, R., Khaled, A.R., Benbernou, N., Rajnavolgyi, E., Muegge, K., and Durum, S.K. (1999). Interleukin-7: physiological roles and mechanisms of action. Cytokine Growth Factor Rev. *10*, 41–60.

Iritani, B.M., Alberola-Ila, J., Forbush, K.A., and Perlmutter, R.M. (1999). Distinct signals mediate maturation and allelic exclusion in lymphocyte progenitors. Immunity *10*, 713–722.

Kincade, P.W., Lee, G., Pietrangeli, C.E., Hayashi, S., and Gimble, J.M. (1989). Cells and molecules that regulate B lymphopoiesis in bone marrow. Ann. Rev. Immunol. *7*, 111–143.

Kitamura, D., Roes, J., Kuhn, R., and Rajewsky, K. (1991). A B cell deficient mouse by targeted disruption of the membrane exon of the immunoglobulin μ chain class. Nature *350*, 423–426.

Koyama, M., Ishihara, K., Karasuyama, H., Cordell, J.L., Iwamoto, A., and Nakamura, T. (1997). CD79α/CD79β heterodimers are expressed on pro-B cell surfaces without associated μ heavy chain. Int. Immunol. *9*, 1767–1772.

Kurosaki, T. (2000). Functional dissection of BCR signaling pathways. Cur. Op. Immunol. *12*, 276–281.

Lai, L., Chen, F., McKenna, S., and Goldschneider, I. (1998). Identification of an IL-7-associated pre-pro-B cell growth-stimulating factor (PPBSF). II. PPBSF is a covalently linked heterodimer of IL-7 and a Mr 30,000 cofactor. J. Immunol. *160*, 2280–2286.

LeBien, T.W. (2000). Fates of human B-cell precursors. Blood *96*, 9–23.

Maki, K., Nagata, K., Kitamura, F., Takemori, T., and Karasuyama, H. (2000). Immunoglobulin beta signaling regulates locus accessibility for ordered immunoglobulin gene rearrangements. J. Exp. Med. *191*, 1333–1340.

Maraskovsky, E., Peschon, J.J., McKenna, H., Teepe, M., and Strasser, A. (1998). Overexpression of Bcl-2 does not rescue impaired B lymphopoiesis in IL-7 receptor-deficient mice but can enhance survival of mature B cells. Int. Immunol. *10*, 1367–1375.

Marshall, A.J., Fleming, H.E., Wu, G.E., and Paige, C.J. (1998). Modulation of the IL-7 dose-response threshold during pro-B cell differentiation is dependent on pre-B cell receptor expression. J. Immunol. *161*, 6038–6045.

Marshall, C.J. (1995). Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. Cell *80*, 179–185.

Mazel, S., Burtrum, D., and Petrie, H.T. (1996). Regulation of cell division cycle progression by bcl-2 expression: a potential mechanism for inhibition of programmed cell death. J. Exp. Med. *183*, 2219–2226.

McKenna, S.D., Chen, F., Lai, L., and Goldschneider, I. (1998). Identification of an IL-7-associated pre-pro-B cell growth-stimulating factor (PPBSF). I. Production of the non-IL-7 component by bone marrow stromal cells from IL-7 gene-deleted mice. J. Immunol. *160*, 2272–2279.

Meffre, E., Casellas, R., and Nussenzweig, M.C. (2000). Antibody regulation of B cell development. Nat. Immunol. *1*, 379–385.

Merchant, M., Caldwell, R.G., and Longnecker, R. (2000). The LMP2A ITAM is essential for providing B cells with development and survival signals in vivo. J. Virol. *74*, 9115–9124.

Mertsching, E., Grawunder, U., Meyer, V., Rolink, T., and Ceredig, R. (1996). Phenotypic and functional analysis of B lymphopoiesis in interleukin-7-transgenic mice: expansion of pro/pre-B cell number and persistence of B lymphocyte development in lymph nodes and spleen. Eur. J. Immunol. *26*, 28–33.

Michie, A.M., Trop, S., Wiest, D.L., and Zuniga-Pflucker, J.C. (1999). Extracellular signal-regulated kinase (ERK) activation by the pre-T cell receptor in developing thymocytes in vivo. J. Exp. Med. *190*, 1647–1656.

Nagaoka, H., Takahashi, Y., Hayashi, R., Nakamura, T., Ishii, K., Matsuda, J., Ogura, A., Shirakata, Y., Karasuyama, H., Sudo, T., et al. (2000). Ras mediates effector pathways responsible for pre-B cell survival, which is essential for the developmental progression to the late pre-B cell stage. J. Exp. Med. *192*, 171–182.

Nagata, K., Nakamura, T., Kitamura, F., Kuramochi, S., Taki, S., Campbell, K.S., and Karasuyama, H. (1997). The Igα/Igβ heterodimer on μ-negative proB cells is competent for transducing signals to induce early B cell differentiation. Immunity *7*, 559–570.

Namen, A., Lupton, S., Hjerrild, K., Wignall, J., Mochizuki, D., Schmierer, A., Mosley, B., March, C., Urdal, D., Gillis, S., et al. (1988). Stimulation of B cell progenitors by cloned murine interleukin-7. Nature *333*, 571–573.

Osmond, D.G., Rico-Vargas, S., Valenzona, H., Fauteux, L., Liu, L., Janani, R., Lu, L., and Jacobsen, K. (1994). Apoptosis and macrophage-mediated cell deletion in the regulation of B lymphopoiesis in mouse bone marrow. Immunol. Rev. *142*, 209–230.

Pages, G., Guerin, S., Grall, D., Bonino, F., Smith, A., Anjuere, F., Auberger, P., and Pouyssegur, J. (1999). Defective thymocyte maturation in p44 MAP kinase (Erk 1) knockout mice. Science *286*, 1374–1377.

Ray, R.J., Stoddart, A., Pennycook, J.L., Huner, H.O., Furlonger, C., Wu, G.E., and Paige, C.J. (1998). Stromal cell independent maturation of IL-7 responsive pro-B cells. J. Immunol. *160*, 5886–5897.

Schotte, P., Declercq, W., Van Huffel, S., Vandenabeele, P., and Beyaert, R. (1999). Non-specific effects of methyl ketone peptide inhibitors of caspases. FEBS Lett. *442*, 117–121.

Shaw, A.C., Swat, W., Davidson, L., and Alt, F.W. (1999a). Induction of Ig light chain gene rearrangement in heavy chain-deficient B cells by activated Ras. Proc. Natl. Acad. Sci. USA *96*, 2239–2243.

Shaw, A.C., Swat, W., Ferrini, R., Davidson, L., and Alt, F.W. (1999b). Activated Ras signals developmental progression of recombinase-activating gene (RAG)-deficient pro-B lymphocytes. J. Exp. Med. *189*, 123–129.

Shinkai, Y., Rathburn, G., Lam, K., Oltz, E.M., Stewart, V., Mendelsohn, M., Charron, J., Datta, M., Young, F., Stall, A.M., and Alt, F. (1992). RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement. Cell *68*, 855–867.

Stephan, R.P., Reilly, C.R., and Witte, P.L. (1998). Impaired ability of bone marrow stromal cells to support B-lymphopoiesis with age. Blood *91*, 75–88.

Stoddart, A., Fleming, H.E., and Paige, C.J. (2001). The role of homotypic interactions in the differentiation of B cell precursors. Eur. J. Immunol. *31*, 1160–1172.

Sutherland, C.L., Heath, A.W., Pelech, S.L., Young, P.R., and Gold, M.R. (1996). Differential activation of the ERK, JNK, and p38 mitogen-activated protein kinases by CD40 and the B cell antigen receptor. J. Immunol. *157*, 3381–3390.

Thornberry, N.A., and Lazebnik, Y. (1998). Caspases: enemies within. Science *281*, 1312–1316.

Tsubata, T., and Reth, M. (1990). The products of the pre-B-cell-specific genes (λ5 and VpreB) and the immunoglobulin μ chain forms a complex that is transported onto the cell surface. J. Exp. Med. *172*, 973–976.

Vairo, G., Innes, K.M., and Adams, J.M. (1996). Bcl-2 has a cell cycle inhibitory function separable from its enhancement of cell survival. Oncogene *13*, 1511–1519.

Valenzona, H.O., Pointer, R., Ceredig, R., and Osmond, D.G. (1996). Prelymphomatous B cell hyperplasia in the bone marrow of interleukin-7 transgenic mice: precursor B cell dynamics, microenvironmental organization and osteolysis. Exp. Hematol. *24*, 1521–1529.

Valenzona, H.O., Dhanoa, S., Finkelman, F.D., and Osmond, D.G. (1998). Exogenous interleukin 7 as a proliferative stimulant of early precursor B cells in mouse bone marrow: efficacy of IL-7 injection, IL-7 infusion and IL-7-anti-IL-7 antibody complexes. Cytokine *10*, 404–412.

Venkitaraman, A.R., Williams, G.T., Dariavach, P., and Neuberger, M.S. (1991). The B-cell antigen receptor of the five immunoglobulin classes. Nature *352*, 777–781.

Vlahos, C.J., Matter, W.F., Hui, K.Y., and Brown, R.F. (1994). A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H–1-benzopyran-4-one (LY294002). J. Biol. Chem. *269*, 5241–5248.

Wei, C., Zeff, R., and Goldschneider, I. (2000). Murine pro-B cells require IL-7 and its receptor complex to up-regulate IL-7Rα, Terminal Deoxynucleotidyltransferase, and cμ expression. J. Immunol. *164*, 1961–1970.

Young, F., Ardman, B., Shinkai, Y., Lansford, R., Blackwell, T.K., Mendelsohn, M., Rolink, A., Melchers, F., and Alt, F.W. (1994). Influence of immunoglobulin heavy and light-chain expression on B cell differentiation. Genes Dev. *8*, 1043–1057.

Young, F., Mizoguchi, E., Bhan, A.K., and Alt, F.W. (1997). Constitutive Bcl-2 expression during immunoglobulin heavy chain-promoted B cell differentiation expands novel precursor B cells. Immunity *6*, 23–33.

Zeng, Y.X., Takahashi, H., Shibata, M., and Hirokawa, K. (1994). JAK3 Janus kinase is involved in interleukin 7 signal pathway. FEBS Lett. *353*, 289–293.